GENOME ANALYSIS OF ENDOPHYTIC *Escherichia coli* USML2 AND ITS *IN PLANTA* ASCENDING MIGRATION WITH GROWTH PROMOTING EFFECT

by

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LIST OF ABBREVIATIONS

NA	Nutrient Agar
NB	Nutrient Broth
BB	Bloomfield broth
WT	Wild-type
PGPE	Plant growth promoting endophyte
PGPB	Plant growth promoting bacteria
PSB	Phosphate solubilizing bacteria
KSB	Potassium solubilizing bacteria
EPS	Extracellular polymeric substances
PCWDE	Plant cell wall degrading enzyme
IAA	Indole-3-acetic acid
ACC	1-aminocyclopropane-1-carboxylate
AHL	N-acylhomoserine lactone
DAT	Day after transplantation
NGS	Next generation sequencing
KEGG	Kyoto encyclopedia of genes and genomes
RAST	Rapid Annotation using Subsystem Technology
GO	Gene ontology
MCP	Methyl–accepting chemotactic proteins
CUP	Chaperone-usher pili
SEM	Scanning electron microscope
TEM	Transmission electron microscope
UPLC	Ultra Performance Liquid Chromatography
HMDS	Hexamethyldisilazane
CDS	Putative protein-coding sequences
CMC	Carboxymethyl cellulose
DNS	3,5-Dinitrosalicylic acid
GI	Genomic island
OGC	Orthologous gene cluster
RE	Restriction enzyme

ANALISIS GENOM ENDOFIT *Escherichia coli* USML2 DAN MIGRASI *IN PLANTA* MENAIKNYA DENGAN KESAN PENGGALAKAN PERTUMBUHAN

ABSTRAK

Penemuan *E. coli* USML2 di dalam tisu daun kelapa sawit (*Elaeis guineensis* Jacq.) yang sihat telah membangkitkan beberapa persoalan mengenai kehadirannya di dalam tanaman dan faedahnya kepada tumbuhan perumah. Oleh yang demikian, keupayaan *E. coli* USML2 berhijrah dari akar ke dalam tisu daun anak benih padi dan menggalakkan pertumbuhan tumbuhan perumah telah dikaji. Berikutan itu, analisis ke atas genom *E. coli* USML2 telah dilakukan untuk mengenalpasti gen yang berkaitan dengan penghijrahan menaik di dalam tanaman dan penggalak pertumbuhan tanaman. Akhir sekali, kepentingan penghijrahan menaik di dalam tanaman ke arah menggalakkan pertumbuhan tanaman juga dikaji. Berdasarkan keputusan, *E. coli* USML2 menakluki permukaan akar dan bahagian dalam tisu batang dan daun dalam masa 24 j inokulasi. Keputusan juga menunjukkan corak jangkitan yang bermula dari penaklukan permukaan akar, pencerobohan ke dalam tisu akar diikuti oleh penghijrahan bakteria ke dalam tisu batang dan daun. Menariknya, inokulasi *E. coli* USML2 menunjukkan peningkatan yang ketara ke atas bilangan daun (33.3%), kandungan klorofil (33.3%), ketinggian pucuk (34.8%) dan berat kering tanaman (90.4%) anak benih padi yang berusia 42 hari berbanding dengan pokok kawalan negatif. Tambahan pula, analisis genom *E. coli* USML2 menunjukkan gen yang mengekod pergerakan bakteria ke arah akar (flagela, kemotaksis), pelekatan kepada akar (pili, penderiaan kuorum, EPS), penaklukan (selulosa dan enzim degradasi pektin) dan penggalak pertumbuhan tanaman

(nitrogen, fosforus, kalium, IAA, ACC, asetoin, siderofor, trehalosa, sulfur, magnesium pengangkut, korismat, kolina, taurina, riboflavin, piridoksin, fenil-propanoid, membran pengangkut). Menariknya, kaedah pulau genomik meramalkan terdapat beberapa gen yang terlibat dalam penghijrahan menaik di dalam tanaman (pili, EPS) dan penggalak pertumbuhan tanaman (pengangkut magnesium, *mgtC*). Selain itu, gen yang terlibat dalam pelekatan kepada akar (protein YdeU tidak dicirikan dan membran luar 'autotransporter barrel-domain' yang mengandungi protein) adalah di antara 24 gen yang unik bagi *E. coli* USML2. Maklumat yang diperolehi daripada analisis genom memudahkan pembinaan mutan *E. coli* USML2 *flhC::Km*^{r} yang tidak boleh bergerak. Mutan menunjukan ketiadaan flagel, pili dan aktiviti kemotaksis. Seterusnya, mengakibatkan kegagalan untuk menakluki tisu-tisu bahagian atas tanaman dan mempamerkan bilangan daun (16.7%), kandungan klorofil (37.4%), ketinggian pucuk (14.5%) dan berat kering tanaman (59.3%) yang lebih rendah berbanding dengan *E. coli* USML2 jenis liar. Ini menunjukan bahawa keupayaan *E. coli* USML2 dalam penghijrahan menaik di dalam tanaman memainkan peranan penting ke arah peningkatan pertumbuhan tumbuhan perumah.

GENOME ANALYSIS OF ENDOPHYTIC *Escherichia coli* USML2 AND ITS *IN PLANTA* ASCENDING MIGRATION WITH GROWTH PROMOTING EFFECT

ABSTRACT

The discovery of *E. coli* USML2 from inner leaf tissues of healthy oil palm (*Elaeis guineensis* Jacq.) raised several questions regarding its presence *in planta* and benefits to the host plant. Therefore, the ability of *E. coli* USML2 to migrate from roots into internal leaf tissues of rice seedlings and promote growth of its host plant was investigated. Subsequently, analysis of *E. coli* USML2 genome was performed to elucidate genes related to *in planta* ascending migration and plant growth promotion. Finally, importance of *in planta* migration towards plant growth promotion was also studied. Based on the results, *E. coli* USML2 colonized the root surface and internal tissues of root, stem and leaf within 24 h of inoculation. Results also indicated an infection pattern initiated from root surface colonization, invasion of the internal root system followed by bacteria migration into the stem and leaf tissues. Interestingly, *E. coli* USML2 inoculation exhibited significant increase of leaf numbers (33.3%), chlorophyll content (33.3%), shoot height (34.8%) and plant dry weight (90.4%) of 42-days-old rice seedlings as compared to the negative control. Furthermore, analysis of *E. coli* USML2 genome revealed genes encoding for bacteria movement towards the root (flagella, chemotaxis), root adhesion (pili, quorum sensing, extracellular polymeric substances), invasion (cellulose and pectin degradation enzymes) and plant growth promotion (nitrogen, phosphorus, potassium, IAA, ACC, acetoin, siderophore, trehalose, sulphur, magnesium transporter, chorismate, choline, taurine, riboflavin, pyridoxine, phenylpropanoids, membrane

transporters). Interestingly, genomic island predicted several *in planta* ascending migration (pili, EPS) and plant growth promotion (magnesium transporter, *mgtC*) genes. Additionally, genes involved in adhesion (uncharacterized protein YdeU and an outer membrane autotransporter barrel-domain containing protein) were among 24 unique genes in *E. coli* USML2. Information provided from genome analysis facilitated construction of a non-motile *E. coli* USML2 *flhC::Km*^{r} mutant. The mutant exhibited absence of flagella, pili and chemotaxis activity. Thus, resulted in failure to invade internal tissues of aerial parts of rice seedlings and exhibited lower leaf numbers (16.7%), chlorophyll content (37.4%), shoot height (14.5%) and plant dry weight (59.3%) compared to the wild-type. This indicated that ability of *E. coli* USML2 in *in planta* ascending migration plays a significant role towards enhancing plant growth of its host plant.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Escherichia coli is a harmless natural inhabitant of the gastrointestinal tract or gut of animals and humans (Ingerson-Mahar and Reid, 2011). Its dispersal into the environment is due to release of fecal matter from the host. This continuous bulk transfers would result into stable population of *E. coli* in secondary habitats such as the soil (Dublan *et al*., 2014). This is why *E. coli* was presumed as not a normal inhabitant of the soil and has been used as an indicator of fecal pollution (Nautiyal *et al*., 2010; Dublan *et al*., 2014). However, discovery of indigenous *E.coli* strains in undisturbed soils of seven uninhabited areas in India with temperatures ranging from -10^{o}C to 45^{o}C demonstrated that *E.coli* is a member of the natural soil biota. Ubiquity of *E.coli* in these diverse soils clearly suggested that *E.coli* should be treated as native soilborne bacteria (Nautiyal *et al*., 2010).

Occurrence of *E. coli* in soils is believed due to its ability to survive, adapt, and actively grow in extra-intestinal environments (Nautiyal *et al*., 2010; Ingerson-Mahar and Reid, 2011; Méric *et al*., 2013; Blount, 2015). Thus, it was hypothesized that persistence of *E. coli* for long periods of time in tropical, subtropical and temperate environments was in response to available nutrients in soil. Substantial nutrient supplies in soil are also provided from excretion of root exudates (Nautiyal *et al*., 2010). Root exudates that consist of carbohydrates, amino acids, organic acids, vitamins and phytohormones act as chemoattractants to which bacteria respond

(Narula *et al*., 2009; Pedraza *et al*., 2009; Berg *et al*., 2013). Thus, attracts broad diversities of soil bacteria to live in the interface between soil and living plant roots known as the rhizosphere (Berg *et al*., 2013; Dudeja and Giri, 2014).

As a consequence, *E. coli* strains have to compete for survival. This is probably the reason why *E. coli* has been frequently found in tissues of plants where the surrounding environment is free from fecal contamination (Dublan *et al*., 2014). Moreover, interior plant tissues offer a less competitive niche for their survival and persistence (Berg *et al*., 2013). Méric *et al.* (2013) reported that presence of *E. coli* in inner parts of leaves is believed due to gain relative protection against adverse conditions present on external surfaces of plants. In fact, interior tissues of the host plant have become a favourable habitat for bacteria capable of plant invasion due to the availability of abundant nutrients and its environmental stability (Ikeda *et al.,* 2010). *E. coli* with the abiliy to live and persist inside the plant or *in planta* are known as endophytes (Hardoim *et al*., 2008).

Endophytes isolated in leaves have been proven to be efficient plant colonizers (Dublan *et al*., 2014). This discovery raised several questions on how it could migrate from the rhizosphere into the root tissues and finally end up in the leaves. Hence, researchers stated that strategies that allow the *in planta* ascending migration are initial root adherence, invasion, colonization and establishment (Böhm *et al*., 2007; Hofkin, 2010; Dublan *et al*., 2014). Chi *et al*. (2005) agreed that endophytes that originally exist in soil would also have ascending action after entering the plant roots. Endophytes usually possess close symbiotic relationships with their host plant (Berg *et al*., 2013). These plant associated bacteria have been described as able to promote growth, health and development of their host plant

(Tahgavi *et al*., 2010). However, knowledge about the effect of endophytic *E. coli* strains on growth of its host plant is very much limited.

In fact, the first and only report on plant growth promoting effects of *E. coli* was demonstrated by Nautiyal *et al*. (2010). In the study, *in planta* colonization of *E.coli* exhibited significant growth promotion of 60-days-old maize (*Zea mays* cv. Arkil) with increased root length, shoot length and plant dry weight when compared with uninoculated seedlings. Hence, it revealed the potential of *E. coli* as a plant growth promoting endophyte. However, *E. coli* strains used by Nautiyal *et al*. (2010) were soilborne bacteria which was introduced anthropically by immersing surface sterilized maize seeds in suspension of *E.coli* cells (8 $\log_{10}$ CFU/mL). Additionally, study by Méric *et al*. (2013) which have isolated *E. coli* strains from interior plant tissues have not reported any information on the effect of its endophytic presence on growth of its host plant.

Since Khairuddin (2012) have isolated *E. coli* USML2 from inner leaf tissues of surface sterilized apparently healthy oil palm (*Elaeis guineensis* Jacq.), therefore this strain was selected as the most suitable candidate to investigate how a plant associated *E. coli* could reached the interior leave tissues, its effect towards growth of the host plant, probable genes involved and the importance of *in planta* ascending migration towards plant growth. Use of *E. coli* in this study is also an advantage due to its rapid cell division rate and rapid adaptation to the environment. Furthermore, *E. coli* only requires simple nutritional prerequisites and has well established genetics and completed genome sequence (Brambilla, 2014). Moreover, to our knowledge, no study has been conducted to discover the *in planta* widespread and potential of indigenous plant-originated *E. coli* on plant growth promotion. In

addition, this is the first report on genome analysis of a plant associated *E. coli*. Thus, this research would aid in understanding the fundamental processes of plant-bacterial interaction.

1.2 Research Objectives

This study was performed to fulfil the following objectives:

1. To confirm the ability of an endophytic *E. coli* USML2 in ascending migration from roots into the leaves of rice seedlings and its influence on plant growth.
2. To determine essential genes in *E. coli* USML2 which are involved in ascending endophytic migration and plant growth promotion.
3. To investigate the effect of motility-impaired *E. coli* USML2 mutant on *in planta* ascending migration and growth promotion of rice seedlings.

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial Endophyte

Microbial endophytes are microorganisms that live internally in healthy plant tissues for all or part of their life cycle without causing apparent harm to the host or its external structure (Hardoim *et al*., 2015). These endophytes ('endo', inside; 'phyte', plant) have been known to be present inside the plant or *in planta* for more than 120 years. They are classified as obligate or facultative based on their life strategies. Obligate endophytes depend strictly on the host plant for growth and survival. However, facultative endophytes can exist not only *in planta* but also outside the host plant. Facultative endophytes can then be grouped into passenger, opportunistic and competent endophytes (Hardoim *et al*., 2008).

Passenger endophytes are endophytes that accidentally enter the plant via colonization of natural wounds or following root invasion by nematodes. Whereas, opportunistic endophytes enter the plants occasionally via root colonization characteristic such as chemotactic response towards root exudates by initially colonizing the rhizoplane, then invading the interior root tissues through cracks formed at sites of lateral root emergence and root tips. However, both passenger and opportunistic endophythes are restricted to the root cortex. In contrast, competent endophytes not only have all properties of an opportunistic endophyte, but are also capable in invasion and spreading inside the whole plant tissues. These competent endophytes successfully colonize *in planta* by actively entering the plant. They have

the capacity to maintain within the plant host even when they are present in high densities (Hardoim *et al*., 2008).

Presence of endophytes has been considered as key determinants of plant health, productivity, community organization and ecosystem functioning (Berg *et al.,* 2013). Their occurrence in the interior tissues is also important by benefiting the plants and improving the yield of agricultural crops (Berg *et al.,* 2013). Endophytes have been found to be ubiquitous, colonizing locally as well as systemically and influencing plant health by suppression of disease, degradation of contaminants and promotion of plant growth (Pedraza *et al.,* 2009; Mohana Kumara *et al.,* 2014). These endophytes can be isolated from surface-sterilized healthy plant tissues (Hallmann *et al.,* 1997; Zinniel *et al.,* 2002; Chi *et al.,* 2005).

2.1.1 Bacterial Endophytes

Researchers have isolated both Gram-positive and Gram-negative bacteria endophytes from tissues of different plant compartments in numerous plant species as presented in Table 2.1. These endophytes consist of both culturable and unculturable bacteria (Taghavi *et al*., 2010; Pedrosa *et al*., 2011). In fact, several different bacterial species have been isolated from a single plant (Zinniel *et al*., 2002; Khairuddin, 2012). Studies of culturable and unculturable microorganisms of the rice roots cv. APO identified 16 phyla or classes of prokaryotic endophytes. Amongst these, the most abundant class was members of Gammaproteobacteria, followed by Alphaproteobacteria (Hardoim, 2015). Its astonishing diversity inside plants could be explained by their ability to enter and persist *in planta* (Hardoim *et al*., 2008).

Table 2.1: Endophytic bacteria isolated from different plant parts in different host plants.

Endophytic Bacteria	Host Plant	Plant Tissue	Reference
Rhizobium sp. *Rhodococcus* sp. *Agrobacterium* sp.	Tomato	Roots	Abbamondi *et al*., 2016
Pseudomonas guinea *Rhizobium giardinii* *Sphingomonas insulae*	*Stellera chamaejasme* L.	Roots	Jin *et al*., 2014
Bacillus megaterium *Burkholderia diazotrophica* *Rhizobium tropici*	Sugar cane	Roots	Paungfoo-Lonhienne *et al*., 2014
Burkholderia phytofirmans PsJN	Onion	Roots	Sessitsch *et al*., 2005
Achromobacter piechaudii	*Sedum plumbizincicola*	Stem	Ma *et al*., 2016
Pseudomonas cremoricolorota *Sphingomonas insulae* *Bacillus safensis*	*Stellera chamaejasme* L.	Stem	Jin *et al*., 2014
Pseudomonas sp. *Microbacterium oleivorans* *Sphingomonas* sp.	*Salix purpurea*	Stem	Gan *et al*., 2014
Herbaspirillum rubrisubalbicans *Burkholderia brasilensis*	Banana	Stem	Cruz *et al*., 2001
Pseudomonas resinovorans *Pantoea dipersa* *Mesorhizobium chacoense*	*Stellera chamaejasme* L.	Leaf	Jin *et al*., 2014
Escherichia coli	Spinach, Rocket Salad	Leaf	Meric *et al*., 2013
Bacillus subtilis *Pseudomonas fluorescens* *Sphingomonas parapaucimobilis*	Switchgrass	Leaf	Gagne-Bourgue *et al*., 2013

Persistence of natural endophytes can vary between 2.0 and 6.0 $\log_{10}$ CFU per gram for alfalfa, sweet corn, sugar beet, squash, cotton, and potato. Similar population levels in plant tissues of tomato and potato were obtained for endophytic bacteria inoculated by root or seed drenching (Zinniel *et al*., 2002). However, concentration of introduced endophytes can reach up to 8.0 $\log_{10}$ CFU/g or higher (Zinniel *et al*., 2002; Nautiyal *et al*., 2010).

Variation in the populations of both indigenous and introduced endophytes actually depends on the plant source, plant age, tissue type, time of sampling and environment (Lamb *et al.,* 1996). These endophytes are able to colonize both intercellular and intracellular spaces of plant roots, stems and leaves (Compant *et al*., 2011; Nair and Padmavathy, 2014; Miliute *et al*., 2015). Generally, bacterial populations are larger in roots and decrease in the stems and leaves (Lamb *et al.,* 1996). However, vital concerns of endophytic colonization in the host plant are the extent of their ascending migration after primary root infection and their population dynamics *in planta*.

2.2 *In planta* Ascending Migration of Endophytes

In planta ascending migration of endophytic bacteria was proposed by early microscopic studies on a native plant endophytic *R. leguminosarum* bv. trifolii which was found in surface sterilized leaf whirls at the stem base of an inoculated gnotobiotic rice seedling (Yanni *et al*., 1997). Following this, researchers suggest that infection process by endophytic bacteria beginning with rhizoplane colonization would subsequently result in ascending migration into the stem base, leaf sheath, and leaves (Chi *et al.,* 2005; Tanuja *et al.,* 2013). Prior to rhizoplane colonization and

invasion, endophytes colonize the rhizosphere which is the volume of soil surrounding plant roots in which bacterial growth is stimulated (Badri and Vivanco, 2009; Taghavi *et al*., 2010). According to Badri and Vivanco (2009), bacteria are more abundant in the rhizosphere due to the presence of root exudates. Therefore, to survive and persist, endophytic bacteria would prefer to migrate into the interior plant tissues for a less competitive niche. Chronological steps involved in ascending migration of endophytic bacteria from roots to inner tissues of aerial plant parts are as shown in Figure 2.1.

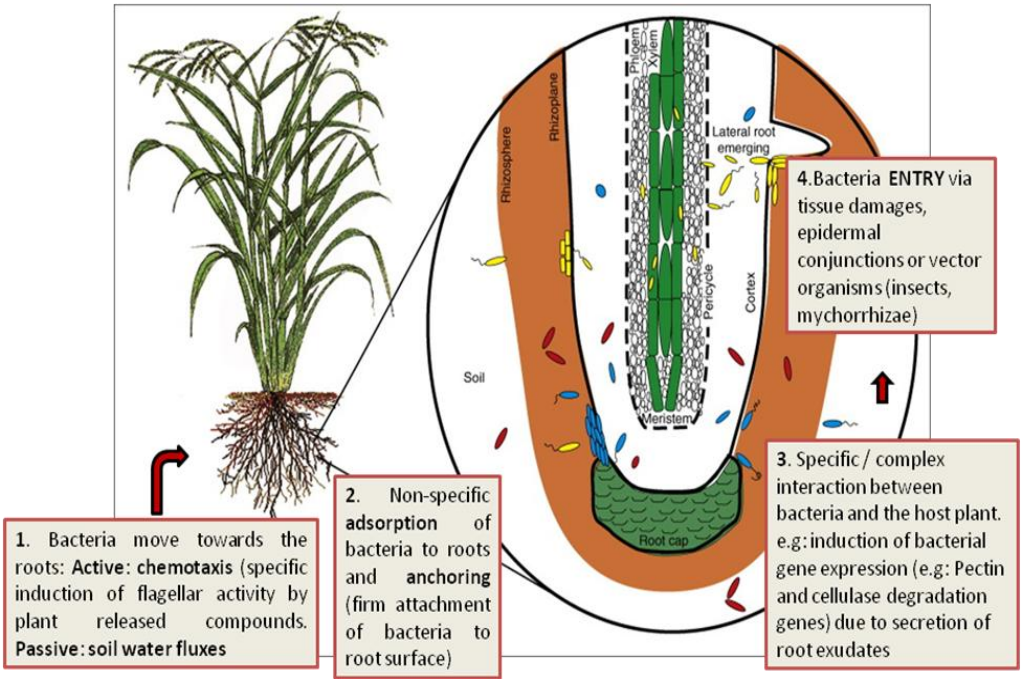

Figure 2.1: Several steps involved in ascending migration of endophytic bacteria from roots to inner tissues of aerial plant parts (Source of picture: Figure 1 in Hardoim *et al*., 2008).

In the preliminary step of ascending endophytic migration, bacteria will move towards the roots passively via soil water fluxes and actively via chemotaxis (Hardoim *et al*., 2008; Taghavi *et al*., 2010). Chemotactic response of the endophytes to nutrients provided on the rhizoplane is facilitated by motility and rotation of the flagella (De Weert *et al*., 2002). Subsequently, the endophytes would attach to the rhizoplane via non-specific adhesion, followed by firm anchoring (Hardoim *et al*., 2008; Taghavi *et al*., 2010). Initial step of attachment is mediated by cell adhesion structure such as pili which is often involved in the transition between motility and irreversible attachment. Consequently, the pili facilitate intercellular interactions through microcolony formation (Berne *et al*., 2015).

During colonization of these microcolonies, the cells are able to communicate via quorum sensing. Production of quorum sensing signals then triggers production of extracellular polymeric substances (EPS) (Bogino *et al*., 2013). Thus, results in firm attachement to the rhizoplane (Taghavi *et al*., 2010). Attachment of the endophytes would then induce numerous bacterial gene expressions due to specific or complex interactions between the bacterium and host plant (Hardoim *et al*., 2008). Finally, bacteria can enter the roots passively at sites of root cracks caused by emergence of lateral roots and actively via root hair zone by production of plant cell wall degrading enzymes such as endoglucanase and endopolygalacturonase (Compant *et al*., 2005; Reinhold-Hurek *et al*., 2006). Endophytes harbouring traits involved in ascending endophytic migration such as motility, chemotaxis and production of plant cell wall degrading enzymes (PCWDEs) are required for successful *in planta* colonization (Compant *et al*., 2010).

2.3 Traits Involved for *In Planta* Ascending Endophytic Migration

2.3.1 Motility

Motility is among the most important trait in bacterial endophytes (Taghavi *et al*., 2010; De Almeida Lopes *et al*., 2016). Motile endophytes can move independently and is likely to play significant role in this early stage of plant-microbe interaction (Vande Broek and Vanderleyden, 1995). Most of motile bacteria move by the use of flagella (Hofkin, 2010; Tambalo *et al*., 2015). Flagella are complex organelles that provide swimming and swarming motilities (Liu and Ochman, 2007). Flagella are thin protein tubes that extend out from the cell surface which enables the cell to spin and move in the environment like a boat's propeller (Hofkin, 2010). Typical bacterial flagella consist of six components which are a basal body, a rotary motor, a switch, a hook, a filament and export apparatus (Liu and Ochman, 2007; Tambalo *et al*., 2015).

Flagellar structure and function has been well studied in *E.coli* and *Salmonella*. They are known as model organisms to study flagella assembly. Flagellar assembly require synthesis of more than 50 genes which vary in their numbers and content among bacteria phyla (Liu and Ochman, 2007; Tambalo *et al*., 2015). The flagellar genes are transcriptionally regulated via combination of mechanisms that create a regulon. Within the regulon, promoters are organized in a three-tier hierarchy classes: class I, class II and class III (Wei *et al.*, 2001; Liu and Ochman, 2007; Tambalo *et al*., 2015).

Class I promoter consists of the master operon, *flhDC*. This master operon is a vital regulatory point in deciding whether to initiate or prevent flagellar

biosynthesis. If either *flhD* or *flhC* gene was disrupted, none of the other genes was expressed. This is because FlhD and FlhC proteins are required to activate transcription of class II flagellar promoters. Genes in the class II promoters encode proteins for structure and assembly of basal body and the hook of the flagellum, as well as for the sigma factor, FliA (σ^{28}). Disruption of genes involved in hook-basal body will still result in transcription of other hook-basal body genes. However, presence of sigma factor, FliA (σ^{28}) would direct transcription of class III genes. These genes are needed for assembly of the flagellar filament, motor activity, chemotaxis and for synthesis of the anti-sigma factor, FlgM which inhibits FliA activity, thus accumulates upon completion of the flagellum (Wei *et al*., 2001; Liu and Ochman, 2007; Raczkowska *et al*., 2011; Tambalo *et al*., 2015).

Flagellum biosynthesis varies in numbers and arrangements depending on species of the bacterium. For example, *Vibrio cholera* and *Campylobacter* have a single flagellum at one end (monotrichous), *Alcaligenes faecalis* has a single flagellum at both ends (amphitrichous), Spirilla has several flagella at one end (lophotrichous) and *E. coli* and *Salmonella* sp. have five to eight flagella over their entire cell surface (peritrichous) (Tambalo *et al*., 2015). Rotation or movement of the flagella will result in motility via "running", smooth swimming or tumbling (Tambalo *et al*., 2015). Bacteria ability to move and be motile is an advantage for endophytes.

Motile ability contributes significantly in movement towards the rhizoplane (Toyota and Ikeda, 1997). In addition, it helps the endophytes to remain in close proximity to the rhizoplane until firmer attachment occured. Glick *et al*. (1999) suggested that plant colonization in soil is an active process that is largely

determined by bacterial motility. Similarly, Toyota and Ikeda (1997) reported that non-motile isolates were poorer root colonizers compared to motile isolates. Potato roots dipped into bacterial suspension of flagella mutants also failed to colonize the roots. Moreover, *Pseudomonas fluorescens* flagellated cells attached more rapidly to glass surfaces compared to non-flagellated cells (Glick *et al*., 1999). Flagellated bacteria also use their flagella to move towards favourable stimulus or chemical attractants (Hofkin, 2010).

2.3.2 Chemotaxis

Bacteria ability to move to its chemoattractants is called chemotaxis (Glick *et al*., 1999; Badri and Vivanco, 2009; Hofkin, 2010). In fact, motility driven by chemotaxis plays an important role in guiding bacteria movement towards rhizoplane colonization, thus mediating plant-bacteria interactions (Dakora and Phillips, 2002; De Weert *et al*., 2002; Szurmant *et al*., 2003; Broeckling *et al*., 2008). This interaction is highly influenced by the substances secreted by plant roots known as root exudates (Narula *et al*., 2009). Increasing evidence proposed that root exudates initiate and modulate dialogue between roots and soil microbes (Badri and Vivanco, 2009). Hence, influences proliferation and survival of root colonizing bacteria (Mukerji *et al*., 2006). This is because root exudates consist of nutrients for the bacteria such as amino acids, sugars, vitamins, organic acids and auxins (Narula *et al*., 2009; Pedraza *et al*., 2009).

Vande Broek and Vanderleyden (1995) reported that sugars such as glucose, galactose, arabinose and xylose are *vir* inducers which are involved in chemotactic response. Similarly, *Agrobacteium tumefaciens* C58 exhibited chemotaxis towards acetosyringone which is a plant phenolic *vir* inducer. Migration towards

acetosyringone was then suggested as probably the initial step in recognition between *A. tumefaciens* and the host plant (Vande Broek and Vanderleyden, 1995). Recognition of chemoattractants involves cell surface chemoreceptors called methyl–accepting chemotactic proteins (MCPs). Prior to binding of a chemotactic ligand, MCPs generate chemotactic signals that are communicated to the flagellar motor via a series of chemotaxis (Che) proteins (Pedraza *et al*., 2009) (Figure 2.2).

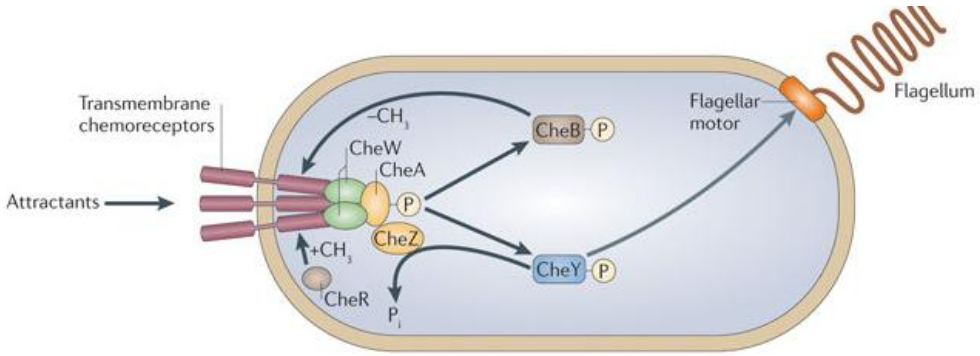

Figure 2.2: Detection of chemoattractants induces flagella-driven chemotaxis which is controlled by transmembrane chemoreceptors and cytoplasmic chemoreceptors (Source: Figure 1 in Porter *et al*., 2011).

Chemotaxis-induced motility is among the strongest deterministic factors for successful endophytic colonization (Compant *et al*., 2010). This was demonstrated by a competitive root colonization experiment where *cheA* mutants exhibited reduced ability to compete with wild-type (WT) strains of *Pseudomonas fluoresc*ens WCS365 (De Weert *et al*., 2002). Similarly, *cheA* mutants of four plant growth promoting bacteria (PGPB) strains of *Pseudomonas fluorescence* was also defective in flagella driven chemotaxis (Narula *et al*., 2009). In addition, mutagenesis of genes involved in chemotaxis sensory proteins for amino acids of *P. fluorescens* Pf0-1 (*ctaA, ctaB, ctaC* genes) were defective in chemotaxis towards 18 naturally-occurring amino acids. These mutants were less competitive than the WT strain in

competitive tomato root colonization assays (Oku *et al*., 2014). Thus, suggests that chemotaxis is considered as an essential mechanism for the successful root colonization (Pedraza *et al*., 2009; Compant *et al*., 2010; Oku *et al*., 2014). Prior to root colonization, close proximity to the plant roots due to chemotaxis would aid in adherence to the target cells on the root surface (Vande Broek and Vanderleyden, 1995).

2.3.3 Bacteria Attachment and Colonization of Plant Roots

Attachment of endophytes to root tissues has been recognized as a prerequisite for further plant-bacteria interactions (Vande Broek and Vanderleyden, 1995). This interaction involves specific bacterial adhesive factors such as pili and exopolysaccharides (Lugtenberg, 2014). Furthermore, bacteria root attachment and colonization is a vital step for an efficient invasion of the plant host (Böhm *et al*., 2007; Hofkin, 2010).

2.3.3(a) Presence of Pili

Pili are filamentous cell appendages that extend out beyond the bacteria cell wall. In general, pili can be divided into four subgroups which are chaperone-usher pili (CUP), type IV pili, alternative chaperone-usher pathway pili and pili assembled by extracellular nucleation-precipitation pathway known as curli (Berne *et al*., 2015). Presence of these adhesive factors allows nonspecific adhesion to specific surfaces such as plant roots (Lugtenberg *et al.*, 2002; Hofkin, 2010). Berne *et al*. (2015) also described pili as functioning like a grappling hook which is crucial in the initial stage of bacterial cell attachment to biotic surfaces. Pili also assist attachment by overcoming initial electrostatic repulsion barrier between the cell and surface (Glick

et al., 1999). Once attached to the surface, it will trigger irreversible attachment (Berne *et al*., 2015). This irreversible attachment influences activation of bacteria gene transcription which provides the bacteria with the ability to spread and proliferate on surfaces (Glick *et al*., 1999).

A number of mutational studies showed that attachment of bacterial cells to the root is a crucial step for subsequent endophytic establishment. Mutant of a rice endophyte (*Azoarcus* sp. BH72) which is impaired in the expression of *pilAB* failed to successfully colonize roots and shoots of rice plants (Dörr *et al*., 1998). Similarly, Maheshwari (2012) also reported impairment in proper adherence and colonization of rice roots by mutant strains of *pilA* and *pilB* which were defective in pilus formation. In addition, *pil*$^{-}$ mutants of *Pseudomonas aeruginosa* exhibited poor surface attachment and failure to move and form cell aggregates (Burdman *et al*., 2011). However, to gain permanent adhesion under variable conditions, production of non-fimbrial adhesion such as extracellular polymeric substances (EPS) would be an advantage (Berne *et al*., 2015).

2.3.3(b) Production of Extracellular Polymeric Substances (EPS)

Extracellular Polymeric Substances (EPS) secreted by bacteria is important in adherence and aggregation of bacterial cells to solid surfaces (Chagnot *et al*., 2013). In fact, EPS itself is defined as extracellular polymeric substances of biological origin that is involved in formation of microbial aggregates. EPS consist of organic marcromolecules formed by polymerization of similar or identical building blocks (Wingender *et al*., 2012). EPS is also known as a non-fimbrial adhesion factor which directly anchors to root surfaces via covalent or non-covalent interactions (Berne *et*

al., 2015). A mutant strain of *Azospirillum brasilense* deficient in EPS showed failure in root anchoring. Hence, EPS was proposed to have a strong role in attachment of bacteria to plant roots (González and Gonzalez-López, 2013).

The significant role of EPS in bacteria attachment to plant roots was in agreement with Meneses *et al*. (2011). In this study, the authors predicted *gumD* gene as responsible for the initial step of EPS production. Subsequently, they demonstrated that EPS production was abolished in *gumD* mutants of *Gluconacetobacter diazotrophicus* PAL5. The *gumD* mutants also failed to attach to inoculated rice roots. Thus suggesting that EPS production may be important during attachment of plant-associated bacteria to the root surface (Meneses *et al*., 2011). Similarly, Rinaudi and González (2009) also reported the importance of EPS in root colonization. In this study, low molecular weight (LMW) succinoglycan or EPS II was shown to be crucial in root colonization of principal roots and root hairs of *Medicago sativa* by EPS-deficient mutants of *Sinorhizobium meliloti*. Absence of EPS might lead to poor root association and host invasion failure. Therefore, it was speculated that the main role of EPS is in positioning the bacteria appropriately on the root surface so that invasion of the plant host can occur (Rinaudi and González, 2009).

2.3.4 Production of Plant Cell Wall Degrading Enzymes

Invasion and colonization of the interior plant host system offers an attractive habitat for endophytes because of the availability of abundant nutrients and its environmental stability (Ikeda *et al.*, 2010). Endophytes can enter the epidermal root tissues passively by penetrating lateral root junctions or actively via production of

plant cell wall degrading enzymes (PCWDE) (Maheshwari, 2012). PCWDE are hydrolytic enzymes involved in degradation of the plant cell wall. These enzymes are required by endophytes to go through the plant cell wall by depolymerisation of the plants primary cell wall structure which is cellulose and pectin (Kubicek *et al*., 2014).

Cellulose is a natural high molecular polymer composed of glucose residues, with cellobiose as the basic coupling unit which accounts for 15-30% dry weight of the primary cell walls. Whereas, pectin which encounters 30% dry weight of the primary cell wall consists of amorphous colloids that have strong hydrophobicity and plasticity. In fact, pectins are crucial for multicell plants to bond neighboring cells together (Chen, 2014). Degradation of cellulose and pectin can be performed by endophytes that are well equipped PCWDE such as endoglucanase and endopolygalacturonase. Production level of PCWDE has been suggested as a factor that could differentiate between endophytes and phytopathogens. This is because endophytes usually produce low levels of PCWDE. In contrast, levels of PCWDE produced by phytopathogens are often deleteriously high (Elbeltagy *et al*., 2000; Maheshwari *et al*., 2012). Production of low levels of PCWDE in endophytes is probably due to avoid triggering the plant defence system. Thus, contributes in entry and spread of endophytes *in planta* (Maheshwari *et al*., 2012).

The role of endoglucanase in endophytic colonization was confirmed by mutation studies where an endoglucanse (*egI*A) gene mutant failed to efficiently invade and systemically colonize the interior plant host (Reinhold-Hurek *et al.*, 2006). Similarly, endoglucanase mutants of *Ralstonia solanacearum* exhibited significantly decreased ability in colonization of tomato stems (Saile *et al*., 1997). Furthermore, mutants of *Erwinia carotovora* subsp. carotovora deficient in either

pehR or *pehS* gene which was required for transcriptional activation of the endopolygalacturonase gene (*pehA*) caused reduction of invasion ability in tobacco seedlings (Flego *et al*., 2000). This finding was in agreement with Huang and Allen (2000) which reported that *Ralstonia solanacearum* mutants lacking an endopolygalacturonase gene (*pehA* gene) decreased its ability in invading stems of tomato plants than the WT. This mutant colonized stems more slowly and had lower mean bacterial populations compared to the WT strain. Thus, the authors suggest that *pehA* gene was required for rapid colonization of the host vascular tissues (Huang and Allen, 2000). Colonization of endophytes *in planta* has been reported by many researchers to have beneficial plant growth promoting effects on the host plant (Hallman *et al*. 1997; Sturz *et al*. 2000; Lugtenberg *et al.,* 2002; Compant *et al*., 2005; Momose *et al*., 2009; Nautiyal *et al*., 2010; Neupane, 2013).

2.4 Plant Growth Promoting Endophyte

Endophytic bacteria with the potential in promoting growth of the host plant are known as plant growth promoting endophyte (PGPE). These PGPEs have been isolated from internal tissues of roots and aerial plant parts (Lindow and Brandl, 2003). Presence of PGPEs in leaves has been speculated due to migration of root endophytes into leaf tissues due to abundant iron and nutrient resources in the above-ground parts of the plant (Bodenhausen *et al*., 2013). Additionally, PGPE with the ability to establish mutualistic relationships with its host plant would exert various beneficial effects to the plant (Hardoim *et al*., 2015) (Table 2.2). In the last few years, the number of identified PGPE has witness a great increase. This includes species of bacteria from the genera *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and

Serratia which have been reported to enhance plant growth (Kloepper *et al*., 1989; Okon and Labandera-Gonzalez, 1994; Glick, 1995; Gururani *et al*., 2012). In fact, PGPE such as *Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum* and *Azomonas* have been widely used as bio-inoculants to promote plant growth and development of agriculture crops (Ahemad and Kibret, 2014). Application of PGPE as inoculants for improving plant growth and yield offers an attractive way to reduce and replace chemical fertilizers, pesticides and supplements (Stefan *et al*., 2008; Ashrafuzzaman *et al*., 2009). This extends to decrease environmental pollution and health risks originating from excessive use of the agrochemical nitrogen (N) fertilizers to achieve enhanced plant growth (Tambalo *et al*., 2015).

Enhancement of plant growth by PGPE have been reported to increase seed germination, rate of radical elongation, seedling vigor, root architecture (length, branching, biovolume, surface area), shoot growth, photosynthetic activity, stomatal conductance, N content of shoot and grain, harvest index, agronomic N fertilizer use efficiency and grain yield of rice (Chi *et al*., 2005). Besides that, inoculation of *Pisum sativum* (Pea) with *Enterobacter cloacae* MSR1 which was isolated from interior tissues of *Medicago sativa* significantly enhanced growth parameters (length and dry weight) of its plant host compared to the non-treated plants (Khalifa *et al*., 2016). Promotion of plant growth by PGPE can be affected by one or more mechanisms that directly influence plant growth promotion at various times during the plant life cycle (Glick *et al*., 1999).

Plant growth promotion by PGPE occurs directly by supplying nutrients through solubilising and mineralising phosphorus from inorganically and organically bound phosphates (Kiers *et al*., 2003; Berg, 2009; Richardson *et al*., 2009). In

addition, many PGPE are able to provide iron and vitamins to plants (Richardson *et al*., 2009). PGPE isolated from tomato were reported harbouring several plant growth promoting traits such as indole-3-acetic acid (IAA), ACC deaminase and siderophore production (Abbamondi *et al*., 2016). Similarly, other researchers agreed that promotion of plant growth was due to presence of plant growth promoting traits in PGPE (Ahemad and Kibret, 2014; El-Sayed *et al*., 2014; Khalifa *et al*., 2016). Therefore, for achieving the most promising plant growth promotion, selection of bacteria comprising suitable plant growth promoting traits is essential (Etesami *et al.*, 2015).

Table 2.2: Effects of PGPE-plant interaction on growth promotion of its host plant (Source: Table 1 in Carvalho *et al*., 2014).

Host Plant	**Bacteria**	**Effect on Growth Promotion**
Rice	*Azoarcus* sp.	Dry weight
	Burkholderia sp.	Root and shoot biomass, grain yield
	Gluconacetobacter diazotrophicus	Dry weight
	Herbaspirillum seropedicae	Root and shoot biomass, yield
	Azobacter sp.	Root length
	Enterobacter sp.	Root length, dry matter yield, grain yield
	Rhizobium leguminosarum bv. Trifolli	Grain yield
Maize	*Burkholderia* sp.	Yield
	Azospirillum brasilense	Yield
	Herbaspirillum seropedicae	Yield
	Gluconacetobacter diazotrophicus	Plant biomass, yield
	Herbaspirillum seropedicae	Dry matter, yield
	Herbaspirillum rubrisubalbicans	Dry matter
	Enterobacter sp.	Root biomass and shoot
	Klebsiella sp.	Biomass
Sorghum	*Azospirillum brasilense*	Lateral root number, root weight, root length
Wheat	*Azospirillum brasilense*	Yield
	Herbaspirillum seropedicae	Plant biomass
Soybean	*Azospirillum brasilense*	Root length

2.5 Plant Growth Promoting Traits

2.5.1 Nitrogen Metabolism

Nitrogen (N) is one of the most vital nutrients for plant development (Carvalho *et al*., 2014; Bouffaud *et al*., 2016). Limited N supply in soil restricts yields of agricultural crops. However, the ability of some beneficial bacteria in fixing atmospheric N_2 to form ammonium provides a promising source of N input in agriculture. In fact, biological nitrogen fixation (BNF) can substitute chemical nitrogen fertilizers (Carvalho *et al*., 2014). Bacteria that are capable in performing BNF such as *Azospirillum, Azotobacter, Gluconoacetobacter diazotrophicus* and *Azocarus* are known as diazotrophs (Ahemad and Kibret, 2014). Unfortunately, not all endophytes have the potential to fix atmospheric N_2. Endophytes incapable in BNF could assimilate nitrate as its source of nitrogen.

Nitrate assimilation initiates active uptake of nitrate by ABC-type transporters. The assimilatory nitrate reductase (encoded by the *nas* genes) converts nitrate to nitrite in the cytoplasm. Finally, nitrite is reduced by assimilatory nitrite reductase to ammonium which is readily available for the plants (Rediers *et al*., 2009). Taghavi *et al*. (2010) reported that a non-nitrogen fixing endophyte (*Enterobacter* sp 638) contained genes required for assimilatory nitrate reduction pathways. Genes for nitrate assimilation are expressed when ammonia and other forms of fixed nitrogen are limiting and nitrate is available (Shapleigh, 2009). However, once the endophytes are in the interior tissues of the host plant, it is uncertain whether plants obtain nitrogen from these endophytic bacteria. Beltran-Garcia *et al*. (2014) stated that endophytic bacteria *in planta* transferred more nitrogen to the host plant and stimulated higher plant biomass than heat-killed

bacteria. In addition, under nutrient limitation, some plants may degrade the endophytes *in planta* as a source of nitrogen by mineralization of dead bacteria cells (Beltran-Garcia *et al*., 2014).

2.5.2 Phosphorus Solubilization

Phosphorus (P) is the most limiting nutrient for crop yields after nitrogen (N). It is particularly essential for almost all major metabolic processes in plants including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Sharma *et al*., 2013). Plants deficient in P usually show inhibited stem and root development, poor flowering and lack of seed and fruit formation (Jin *et al*., 2014). This is because P is one of the major essential macronutrients for plants. It is applied to soil in the form of phosphatic fertilizers. However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants (Chen *et al*., 2005). According to Sharma *et al*. (2013), only 0.1% of the P content in soil (~0.05%; w/w) is available to the plant due to poor solubility. Therefore, PGPE capable in solubilization of P are considered as important solubilizers of insoluble inorganic phosphate also known as phosphate solubilizing bacteria (PSB) (Zhu *et al*., 2011).

Numerous PSB have been isolated since the beginning of the first century. This includes *Arthrobacter, Azotobacter, Pseudomonas, Agrobacterium, Bacillus, Erwinia, Flavobacterium, Enterobacter, Micrococcus, Bradyrhizobium, Salmonella, Alcaligenes, Chromobacterium, Serratia, Streptomyces, Thiobacillus* and *Escherichia*. These PSB are involved in a range of processes that affect the transformation of soil P and are thus an integral part of the soil P cycle. In particular,

they are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization (Hilda and Fraga, 1999; Chen *et al*., 2006). The ability to convert insoluble forms of phosphorus to an accessible form is a significant trait in PGPE for increasing plant growth and yields (Chen *et al*., 2006; Sharma *et al*., 2013). Application of PSBs as inoculants has been reported to increase the P uptake by plants. However, mutants of *Rhizobium* sp. MR-54 deficient in mineral phosphate solubilization exhibited reduced plant growth compared to the WT strain under green house conditions in Leonard jar apparatus. Thus, indicated that phosphate solubilization is essential in growth and development of the plant host (Dahale *et al*., 2016).

2.5.3 Potassium Solubilization

Potassium (K) is the third most essential macronutrient for plant growth and development (Prajapati *et al*., 2013). In fact, plants require K in large quantities. This is because K is involved in promotion of root growth, stronger stem and increases resistance to cold and water stress. Besides that, it contributes in improvement of the crop quality, reduces disease and pest incidence by enhancing crop resistance (Bagyalakshmi *et al*., 2012). Pottasium is also important in activation of several metabolic processes which includes photosynthesis, protein synthesis and enzyme activation (Prajapati *et al*., 2013). Thus, plays a pivotal role to plant growth, yield, quality and stress (Bagyalakshmi *et al*., 2012). Deficiency of K in plants causes slow growth, burned appearance of the leaf edges and incomplete root development (Zhang and Kong, 2014).